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(71) Applicant: THE GOVERNMENT OF THE UNITED STATES OF AMERICA, as represented by THE SECRETARY OF THE DEPARTMENT OF HEALTH AND HUMAN SERVICES [US/US]; Office of Technology Transfer, Box OTT, Bethesda, MD 20892-9902 (US).

(72) Inventors: DONOVAN, Peter, J. ; 6144 Mt. Phillip Road, Frederick, MD 21702 (US). RESNICK, James, L. ; 421 Navaho Drive, Frederick, MD 21701 (US).

(74) Agents: ALTMAN, Daniel, E. et al.; Knobbe, Martens, Olson & Bear, 620 Newport Center Drive, 16th floor, Newport Beach, CA 92660 (US).

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(57) Abstract

The invention includes a method of culturing primordial germ cells from an animal *in vitro* for a period of time longer than seven days. This method includes growing primordial germ cells in the presence of a fibroblast growth factor and leukemia inhibitory factor. The invention also includes the resulting cell cultures and transgenic and chimeric animals produced with the aid of such cultures.

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LONG-TERM PROLIFERATION OF PRIMORDIAL GERM CELLS

Background of the Invention

In all vertebrate groups, the progenitors of the germ line, the primordial germ cells (PGCs) arise extragonadally and move to the developing gonad early in embryonic development. Entry of the PGCs into the gonad results in several well characterized changes in the germ cell. In the male, PGCs enter mitotic arrest, whereas in the female they enter the first meiotic prophase. Loss of a number of antigenic determinants from the germ cell surface also occurs in both sexes. The decreasing ability of successively older embryonic mouse gonads to generate germ cell derived tumors (teratomas) when grafted into the testis capsule of syngeneic adult hosts is also suggestive of major changes in germ cell phenotype.

In mouse embryos, primordial germ cells (PGCs) can be identified histochemically, due to their high content of alkaline phosphatase, at 8.5 days post coitum (dpc) at the root of the allantois. At subsequent developmental stages they are found in the hindgut (9.5 dpc), the hindgut mesentery (9.5-11.5 dpc), and the dorsal body wall (from 10.5 dpc). By 12.5 dpc the PGCs are largely confined to the developing gonad. PGC ultrastructure, and the movements of cells identified as PGCs in squash preparations suggest that migration to the gonad involves active locomotion.

Detailed analysis of PGC behavior *in vitro* has previously been hindered by lack of a suitable substrate for growth. Isolated mouse gonadal germ cells have consistently been shown to be nonadherent to glass, plastic, or gelatin-coated substrata. In explant cultures, however, PGCs do adhere to outgrowths of embryonic somatic cells, and furthermore, isolated PGCs adhere to embryo-derived cell lines.

The observed locomotory behavior of pregonadal PGCs supports the proposal that colonization of the gonad anlagen is brought about by active movement of PGCs. Furthermore, it suggests that this locomotion is invasive. The changes in morphology of PGCs isolated at progressively later stages implies an alteration in PGC behavior *in vivo* after gonad colonization. This indicates a major change in phenotype during this process.

Murine primordial germ cells (PGCs) are first identifiable as a population of approximately eight alkaline phosphatase (APase) positive cells in the 7.0 days post coitum embryo. During the subsequent six days of development they proliferate to give rise to the approximately 25,000 cells that establish the meiotic population. Steel factor (SLF) is required for PGC survival both *in vivo* (Bennett, D., *J. Morph.*, **98**, 199-233 (1956)) and *in vitro* (Dolci, S., et al., *Nature*, **352**, 809-811 (1991)) and together with Leukemia Inhibitory Factor (LIF) stimulates PGC proliferation *in vitro* (Matsui, Y., et al., *Nature*, **353**, 750-752 (1991)).

In feeder-dependent culture, PGCs will proliferate for up to 7 days, but their numbers eventually decline and their proliferative capacity is only a small fraction of that seen *in vivo*.

Therefore, it would be advantageous to maintain the PGCs for longer than 7 days in culture. A long-term culture of PGCs would allow researchers the necessary time to carry out their protocols before cell death.

Summary of the Invention

One embodiment of the present invention is a method of culturing primordial germ cells from an animal *in vitro* for a period of time longer than seven days. This method comprises growing the primordial germ cells in the presence of a fibroblast growth factor and leukemia inhibitory factor. In this method the fibroblast growth factor most preferably is basic fibroblast growth factor (bFGF). As part of this embodiment, the primordial germ cells can advantageously be grown in the presence of steel factor, and most preferably, the primordial germ cells are grown in the presence of both soluble steel factor and membrane-bound steel factor.

A further preferred embodiment of the present invention includes growing the primordial germ cells on a layer of feeder cells, with the feeder cells advantageously being STO cells. In this embodiment the feeder cells can provide the source of steel factor, and preferably provide a source of both soluble steel factor and membrane-bound steel factor. In an even more preferred embodiment, the feeder cells are mitotically inactive fibroblasts. Advantageously, these fibroblasts are murine STO cells or an analogue thereof from a non-murine animal. Most preferably however, the steel factor, fibroblast growth factor and leukemia inhibitory factor are from the same animal as the primordial germ cells. Alternatively, these factors can most advantageously be derived from an animal more highly evolved than the animal from which the primordial germ cells are derived.

In one embodiment of this preferred method, the primordial germ cells are isolated by dissection from embryos and the animal is selected from the group consisting of a chicken, a cow, a sheep and a pig. In a further embodiment of this invention, the primordial germ cells can be derived from a mouse. The primordial germ cells can also be advantageously grown on feeder cells to form a culture. Additionally the growth of these primordial germ cells can comprise splitting the culture and growing the primordial germ cells on fresh feeder cells. In a further preferred embodiment, the primordial germ cells are grown in the presence of cholera toxin.

A different aspect of the present invention includes a culture of primordial germ cells that will grow for a period longer than seven days. Most preferably the culture will grow for a period longer than fourteen days. Even more preferably the culture is a continuous culture that will grow for an indefinite period. An even more preferable culture can advantageously comprise basic fibroblast growth factor, and even more preferably the culture additionally comprises steel factor. In this embodiment, the culture can most preferably comprise both

soluble steel factor and membrane-bound steel factor, and even still more preferably can also comprise leukemia inhibitory factor. In this embodiment the culture can advantageously include the primordial germ cells on a layer of feeder cells, while the feeder cells are most advantageously mitotically inactive fibroblasts.

Yet another embodiment of the present invention is a method of producing a chimeric animal, including growing primordial germ cells in the presence of a fibroblast growth factor and leukemia inhibitory factor to form a primordial germ cell culture, isolating one or more cells from the culture, inserting the one or more cells into a developing embryo, and growing an animal from the embryo. One preferable embodiment of this embodiment includes the animal being selected from the group consisting of a cow, a pig, a chicken, a mouse and a sheep.

Still another embodiment of the present invention is a method of producing a transgenic animal, including growing primordial germ cells having a given genotype in the presence of a fibroblast growth factor and leukemia inhibitory factor to form a primordial germ cell culture, isolating one or more cells from the culture, inserting the one or more cells into a developing embryo, growing an animal from the embryo, wherein the one or more cells contribute to the germline of the animal, and allowing the animal to reproduce wherein the given genotype is present in the resulting offspring. One preferred embodiment of this method includes the animal being selected from the group consisting of a cow, a pig, a mouse and a sheep. A further preferred embodiment of the present invention includes inserting one or more cells into a sterile animal with no germ cells. In this preferred embodiment, the injection is advantageously into one or more cells of a post-implantation embryo. An even more preferred embodiment includes injecting through the uterus of the animal in which the previous growing step occurs.

Description of the Figures

Figure 1. Analysis of PGC proliferation in culture.

The effect of mLIF concentration on PGC proliferation in culture. The number of PGCs present after 1 and 3 days of culture on confluent STO cell feeder layers. Bars represent the mean plus/minus the standard deviation of five replicate cultures. Bars represent the mean plus/minus the standard deviation of five replicate cultures. Each experiment was done four times.

Figure 2. The effect of bFGF concentration on PGC Proliferation in culture. The number of PGCs present after 1 and 3 days of culture on confluent STO cell feeder layers. Bars represent the mean plus/minus the standard deviation of vie replicate cultures. Each experiment was done four times.

Detailed Description

We have discovered that fibroblast growth factor, and especially basic Fibroblast Growth Factor (bFGF) stimulates PGC proliferation *in vitro*. In addition, bFGF, in the presence of Steel Factor (SLF) and Leukemia Inhibitory Factor (LIF), stimulates long-term proliferation of PGCs leading to the derivation of large colonies of cells. These colonies of cells (we term embryonic germ (EG) cells) resemble embryonic stem (ES) cells. ES cells are pluripotent cells derived from pre-implantation embryos or feeder-dependent embryonal carcinoma (EC) cells. These embryonal carcinoma cells are pluripotent stem cells from PGC-derived tumors (teratomas). Our discovery provides the first system for long-term culture of PGCs or PGC-derived cell lines.

We have previously shown that the transmembrane form of Steel Factor is required for PGC survival in culture. Moreover, we have observed that a soluble factor produced by STO cells can stimulate PGC proliferation. STO cells are mitotically inactive fibroblast cells; such cells, or analgous cells, can be isolated from a wide variety of animals. Studies by Hogan and her colleagues have shown that this factor is probably LIF. *Nature*, **353**:750-752 (1991). LIF exists as both a diffusible form (LIF-D) and a matrix-associated form (LIF-M). Although STO cells produce LIF, we have found that addition of recombinant, murine LIF (mLIF) to the culture medium enhances murine PGC proliferation on STO cells in a dose-dependent manner, with a peak response around 1000 units/ml (see Figure 1). STO cells also produce steel factor in at least two forms: a soluble form and a membrane-bound form.

Example 1
Growth of PGCs in the presence of mLIF

A. PGC Isolation

Embryos were derived from matings of the outbred mouse strain MF1 (OLAC), the finding of a vaginal plug was denoted day 0 of pregnancy. Germ-cell-containing fragments of embryos were isolated by sterile dissection in Ca^{++}/Mg^{++}-free phosphate buffered saline (PBS), washed in several changes of PBS, and triturated repeatedly in a small volume of PBS to yield a single-cell suspension. Small aliquots of this germ-cell-containing suspension were added to preformed STO feeder layers at one embryo equivalent per feeder layer and allowed to attach for 16 hr.

B. Cell Culture

STO cells were routinely grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS), glutamine (4 Mm), penicillin, and streptomycin (DME). The cells were harvested just prior to confluence by trypsinization (0.05% trypsin, 0.02% EDTA in PBS), washed, and irradiated (600 rads) in a cesium gamma source. Irradiated STO cells were adjusted to 5×10^4 cells per ml in DME and 200 μl per well were added to 96 well plates (Costar) or 2 ml to 30 mm diameter tissue culture petri dishes. At this plating density a

subconfluent monolayer is formed, the cells gradually spreading out, leaving some areas of substrate exposed. Germ-cell-containing cell suspensions prepared as described above were added to irradiated subconfluent STO cell feeder layers 24-48 hr after plating.

C. Growth of PGCs in the presence of mLIF

Figure 1 shows the effect of 1000 U/ml (hollow), 2000 U/ml (hatched), and 5000 U/ml (checkered) of mLIF on PGC proliferation in culture. Media with only DMEM (solid) was the negative control. The horizontal axis represents the number of PGCs present after 1 and 3 days of culture on confluent STO cell feeder cells plus the mLIF. Each bar represents the mean plus/minus the standard deviation of five replicate cultures. Each experiment was repeated four times.

The results of this example show that a concentration of 1000 U/ml of mLIF leads to the greatest proliferation of PGCs *in vitro*.

Because of its ability to stimulate both LIF-D and LIF-M production by feeder cells, we analyzed the effect of bFGF alone on PGC proliferation, as in Example 2.

Example 2

Effects of bFGF alone on PGCs

Aliquots of bFGF in concentrations of 1 ng/ml, 5 ng/ml and 10 ng/ml, were added to cultures of PGCs on STO cells *in vitro* (Figure 2) similar to the methods described above. The bFGF stimulated dose-dependent proliferation of PGCs with a peak response of approximately 1 ng/ml.

At their optimal concentrations, both LIF and bFGF stimulated PGC proliferation but the number of viable PGCs continued to decline after 5 days of incubation on STO cells. This result was consistent with our previous observations which showed that PGCs could not be maintained in culture for longer than 7 days. After demonstrating that bFGF could increase the lifespan of PGCs *in vitro*, we tested the combination of bFGF and mLIF on murine primordial germ cells. Surprisingly, we observed continued growth of some PGCs in the presence of LIF and bFGF leading to the formation of increasingly large PGC colonies.

Example 3

Incubation of PGCs with bFGF and Exogenous LIF

Cultures of PGCs were established from 8.5 days post coitus (dpc) or 11.5 dpc embryos as described above. PGCs were identified by alkaline phosphatase histochemistry. Briefly, histochemical and immunocytochemical staining was carried out on tissue fixed for 1 hr, or cells fixed for 15 min, at room temperature in 4% para-formaldehyde in PBS. Alkaline phosphatase staining was performed at room temperature in 25 Mm Tris-maleate buffer (pH 9.0) containing 0.15 M NaCl. Tissue blocks, frozen sections, or cell cultures were incubated for 15 min in a solution of 0.4 mg/ml sodium alpha naphthyl phosphate, 1 mg/ml Fast Red TR salt, and 4 mM $MgCl_2$. Enzyme activity was localized by the appearance of a red reaction product

which was inhibited, in control experiments, by the presence of 1 mM levamisole, a specific inhibitor of the enzyme. We evaluated the proliferation of PGCs incubated with 1000 U/ml bFGF and 1 mg/ml LIF on STO feeder cells. The PGCs grew and formed EG type cell colonies. We took the following photographs, which are not provided, and made the following observations:

a. PGCs, identified by APase activity one day after isolation from 8.5 dpc embryos and plating onto STO cell feeder layers in bFGF and LIF. PGCs existed as single cells, with no evidence of clumps or cell colonies.

b. Three days after isolation, PGCs were beginning to form small colonies, either by clonal division or aggregation. Some single PGCs were still identifiable.

c. Five days after isolation, PGCs were found in increasingly larger colonies. Many small colonies were found clustered near each other.

d. Small colony of EG cells growing as a cellular monolayer on top of the STO feeder layer.

e. Two colonies of EG cells. The smaller colony was growing as a monolayer, while the large colony had become multilayered.

f. Large, several hundred micrometer, multilayered EG cell colony identified 9 days after plating PGCs onto confluent STO cell feeder layers.

In the embryo, PGCs demonstrate APase activity from 7.0 dpc to 15.5 dpc. Another classical marker of PGCs is the carbohydrate differentiation antigen SSEA-1, which is expressed on the PGC surface from 8.5 dpc to 14.5 dpc (Fox N., et al., *Dev. Biol.*, **83**, 391-398 (1981)) . Both APase and SSEA-1 are definitive markers of PGCs in these cultures. Like PGCs, colonies of EG cells continued to have APase activity and to express the SSEA-1 antigen, as demonstrated in the following example.

Example 4

Antigenic expression of PGCs

Ascitic fluids of the two monoclonal antibodies were used at 1:100 dilution and the rhodamine-labeled rabbit anti-mouse second antibody (Nordic) at 1:50.

Cultures of PGCs were established as described above. For immunocytochemistry protocols, cultures were fixed with 4% para-formaldehyde in phosphate buffered saline (PBS), washed three times with PBS, and then incubated with primary (anti-SSEA-1) antibody (diluted in 10% heat inactivated normal goat serum) for 1 hour at room temperature. The cultures were then washed three times with PBS and incubated with the peroxidase-conjugated secondary (goat anti-mouse polyvalent immunoglobulin 1:50 dilution, NORDIC) antibody for 30 minutes at room temperature. The first and second antibodies were applied for 1 hr after blocking nonspecific binding sites with 10% heat inactivated normal rabbit serum in 4% bovine serum

albumin in PBS. Extensive washing with PBS followed each antibody application. Positive antibody staining was identified by peroxidase histochemistry using DAB, hydrogen peroxide, cobalt and nickel chloride. Positive cells were stained dark brown in color.

Antigenic and morphologic characterization of PGC-derived EG cells was also evaluated. We took the following photographs, which are not provided, and made the following observations:

a. Surface staining of an EG cell colony with an anti-SSEA-1 antibody (IgM). Positive staining was detected as a peroxidase precipitate. STO cells and other embryonic somatic cells were negative.

b. Surface staining of EG cell colony with an isotype matched (IgM) control antibody. No peroxidase staining was observed.

c. Phase contrast micrograph of an EG cell colony growing on top of the STO cell feeder layer. Large colonies of EG cells closely resembled feeder-dependent EC cells or ES cells grown on STO feeder layers.

We are presently unsure how the EG cells we have derived by the combination of LIF, SF and bFGF relate to normal PGCs *in vivo*. While not wishing to be bound by any particular explanation for this effect, three possibilities seem likely: (1) EG cells are simply proliferating PGCs; (2) EG cells are PGCs that have become immortalized in culture; or (3) EG cells are a differentiated or de-differentiated derivative of PGCs. Regardless of whichever of these possibilities proves to be correct these cells represent an invaluable reagent for the analysis of PGC development and can be used to study such key issues as germ line imprinting, recombination, sexual differentiation and pluripotency.

Two other cell types that have APase activity and express the SSEA-1 antigen are pluripotent ES and EC cells. Interestingly, as discussed above, large colonies of EG cells closely resemble feeder-dependent EC cells or ES cells grown on STO feeder layers. How EG cells are related to EC cells or ES cells remains to be determined.

We believe that techniques similar to the foregoing can be used to create continuous cultures of primordial germ cells from a wide variety of animals, including chickens, pigs, cows and sheep. Thus, we demonstrated the utility of these techniques in cows, as described in Example 5.

Example 5

Culture of Primordial Germ Cells from Bovine Embryos

A pure-bred Simmental Cow was superovulated with follicle stimulating hormone (FSH) AUSA Superov over a four day period. 2.5 ml FSH twice daily was given the first day, 2.0 ml twice daily on each of the second and third days, and 1.0 ml twice daily on the fourth day. This provided 75 units of FSH over a four day period. On the fourth day, the animal was

given prostaglandin F2α as Estrumate (Cloprostenol); 4.0 ml in the morning and 2.0 ml in the afternoon. On the fifth day, the animal was in heat and was mated with two Simmental bulls. Thirty-five days later the animal was examined by ultrasound to confirm the pregnancy, to count the number of embryos and to determine their viability. The paralumbar fossa of the animal was clipped and surgically prepped. Local anaesthetic was Lidocaine. The abdominal cavity was entered surgically and the uterine horn was exteriorized. Each embryo was located and isolated surgically. The embryos were placed into cooled phosphate buffered saline (PBS) and the genital ridges were identified and dissected out. The genital ridges were dissociated in trypsin/EDTA and the resultant cell suspension was plated onto confluent monolayers of irradiated STO cells. Cultures were fed in DMEM plus 15% fetal bovine serum, Na pyruvate, L-glutamine, penicillin and streptomycin with added bFGF and LIF. To some of these cultures, cholera toxin was also added. Separate cultures were established containing either mouse or human recombinant LIF. After five days, a duplicate culture was fixed and stained for alkaline phosphatase activity to identify primordial germ cells (PGCs). Many PGCs were identified. A duplicate culture was passaged onto fresh feeders by trypsinization and continuously maintained. Cells with the morphology of EG cells continued to grow in these cultures.

Unexpectedly, we found that cholera toxin was a potent germ cell mitogen. We also found that both the recombinant human and murine LIF functioned effectively. However, we believe that native human LIF functions effectively in bovine cells while native murine LIF will function much less effectively. Thus, we believe that factors from the same animal or from an animal more highly evolved will function effectively in the continuous cell line system. From these results, we believe that it is the glycosylation pattern of the various factors that determines its effectiveness. Similarly, STO cells from the same animal as the primordial germ cells will serve as effective feeder cells, while STO cells or their analogues from an animal less highly evolved will not function as well. However, STO cells from a more highly evolved animal will provide effective continuous culture. STO cells or quite similar analogs can be derived from a very large number of animals and can function as effective feeder cells for cultures of primordial germ cells from many such animals.

A major roadblock to our understanding of the development of the mammalian germ line heretofore has been the inability to culture PGCs for significant periods of time. The failure of PGCs to proliferate *in vitro* at the same rate as they do *in vivo* suggests the requirement for factors other than just SLF and LIF. We have demonstrated the first example of long-term proliferation of PGCs and demonstrated a role for bFGF in regulating PGC proliferation. We believe that basic FGF acts through its ability to stimulate LIF production in the feeder cells or, alternatively, that it binds directly to PGCs via an FGF receptor (FGF-R). Whether bFGF or LIF are expressed in the developing gonad or whether PGCs express an FGF-R or a LIF receptor

remain to be determined. Nevertheless, the ability of bFGF and LIF to stimulate long-term PGC proliferation provides a unique tool for the analysis of PGC development and for the creation of chimeric an transgenic animals.

Chimeric and transgenic animals of many sorts, including pigs, chickens, cows, sheep and mice, can be made from these cell lines. In order to produce a chimeric animal, primordial germ cells are grown in the presence of a fibroblast growth factor and leukemia inhibitory factor as described above to form a primordial germ cell culture. These factors are present throughout a wide range of animals, including the pigs, chickens, cows, sheep and mice discussed above. We have found that these factors work best if they are from the same animal as the animal from which the cells are derived or if the factors are from an animal more highly evolved than the animal from which the cells are derived. Thus, the human factors will function effectively with cells from most animals. However, the murine factors will function effectively only with cells derived from mice. One or more cells from the culture are then isolated and inserted into a developing embryo of the same animal as the cells from which the cells are derived. The blastocysts or other suitable stage embryos can be used. A whole chimeric animal can then be derived from this embryo by growing the embryo *in utero*.

Transgenic animals can also be created by following the procedure described above for creating a chimeric animal. For transgenic animals, the one or more cells from the culture should contribute to the germline of the resulting animal. Thus, when this animal is allowed to reproduce, the genotype of the primordial germ cells is present in the resulting offspring. In order to facilitate the inclusion of the cells from the culture into the germline, the cells can be inserted, such as through sterile injection, into a sterile embryo lacking germ cells. The cells from the culture can also be inserted into a post-implantation embryo, such as through the uterus of the animal in which the embryo is growing.

WHAT IS CLAIMED IS:

1. A method of culturing primordial germ cells from an animal *in vitro* for a period of time longer than seven days, said method comprising growing said primordial germ cells in the presence of a fibroblast growth factor and leukemia inhibitory factor.

2. The method of Claim 1, wherein said fibroblast growth factor comprises basic fibroblast growth factor.

3. The method of Claim 1, wherein said primordial germ cells are grown in the presence of a steel factor.

4. The method of Claim 3, wherein said primordial germ cells are grown in the presence of both soluble steel factor and membrane-bound steel factor.

5. The method of Claim 1, wherein said primordial germ cells are grown on a layer of feeder cells.

6. The method of Claim 5, wherein said feeder cells provide a source of steel factor.

7. The method of Claim 6, wherein said feeder cells provide a source of both soluble steel factor and membrane-bound steel factor.

8. The method of Claim 5, wherein said feeder cells comprise mitotically inactive fibroblasts.

9. The method of Claim 5, wherein said feeder cells comprise murine STO cells or an analogue thereof from a non-murine animal.

10. The method of Claim 1, wherein said steel factor, fibroblast growth factor and leukemia inhibitory factor are from the same animal as said primordial germ cells or from an animal more highly evolved than the animal from which said primordial germ cells are derived.

11. The method of Claim 1, wherein said primordial germ cells are isolated by dissection from embryos.

12. The method of Claim 1, wherein said animal is selected from the group consisting of a chicken, a cow, a sheep and a pig.

13. The method of Claim 1, wherein said animal is a mouse.

14. The method of Claim 5, wherein the cells are grown on feeder cells to form a culture, additionally comprising splitting said culture and growing said primordial germ cells on fresh feeder cells.

15. The method of Claim 1, wherein said primordial germ cells are grown in the presence of cholera toxin.

16. A culture of primordial germ cells that will grow for a period longer than seven days.

17. The culture of Claim 16, wherein said culture will grow for a period longer than fourteen days.

18. The culture of Claim 17, wherein said culture is a continuous culture that will grow for an indefinite period.

19. The culture of Claim 16, wherein said culture comprises basic fibroblast growth factor.

20. The culture of Claim 19, wherein said culture additionally comprises steel factor.

21. The culture of Claim 20, wherein said culture comprises soluble steel factor and membrane-bound steel factor.

22. The culture of Claim 19, wherein said culture comprises leukemia inhibitory factor.

23. The culture of Claim 16, wherein said culture comprises said primordial germ cells on a layer of feeder cells.

24. The culture of Claim 23, wherein said feeder cells comprise mitotically inactive fibroblasts.

25. A method of producing a chimeric animal, comprising:

growing primordial germ cells in the presence of a fibroblast growth factor and leukemia inhibitory factor to form a primordial germ cell culture;

isolating one or more cells from said culture;

inserting said one or more cells into a developing embryo; and

growing an animal from said embryo.

26. The method of Claim 25, wherein said animal is selected from the group consisting of a cow, a pig, a chicken and a sheep.

27. The method of Claim 25, wherein said animal is a mouse.

28. A method of producing a transgenic animal, comprising:

growing primordial germ cells having a given genotype in the presence of a fibroblast growth factor and leukemia inhibitory factor to form a primordial germ cell culture;

isolating one or more cells from said culture;

inserting said one or more cells into a developing embryo;

growing an animal from said embryo, wherein said one or more cells contribute to the germline of said animal; and

allowing said animal to reproduce wherein said given genotype is present in the resulting offspring.

29. The method of Claim 28, wherein said animal is selected from the group consisting of a cow, a pig and a sheep.

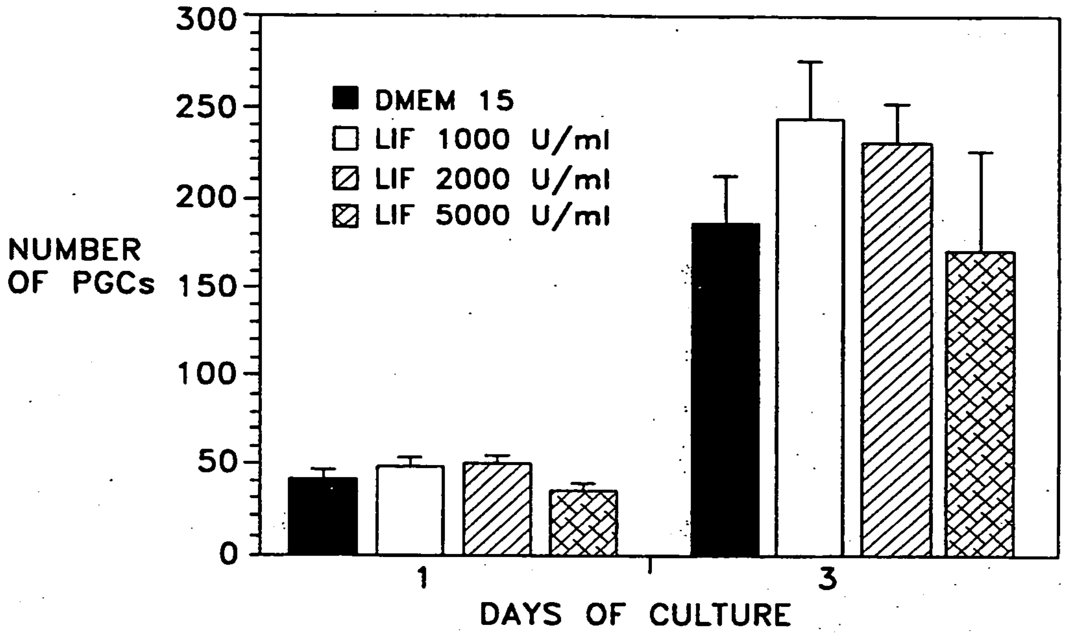
30. The method of Claim 28, wherein said animal is a mouse.

31. The method of Claim 28, wherein the inserting step comprises inserting said one or more cells into a sterile animal with no germ cells.

32. The method of Claim 28, wherein the inserting step comprises injecting said one or more cells into a post-implantation embryo.

33. The method of Claim 32, wherein the injecting step comprises injecting through the uterus of an animal in which the growing step occurs.

FIG. 1


300
250
200
150
100
50
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NUMBER OF PGCs
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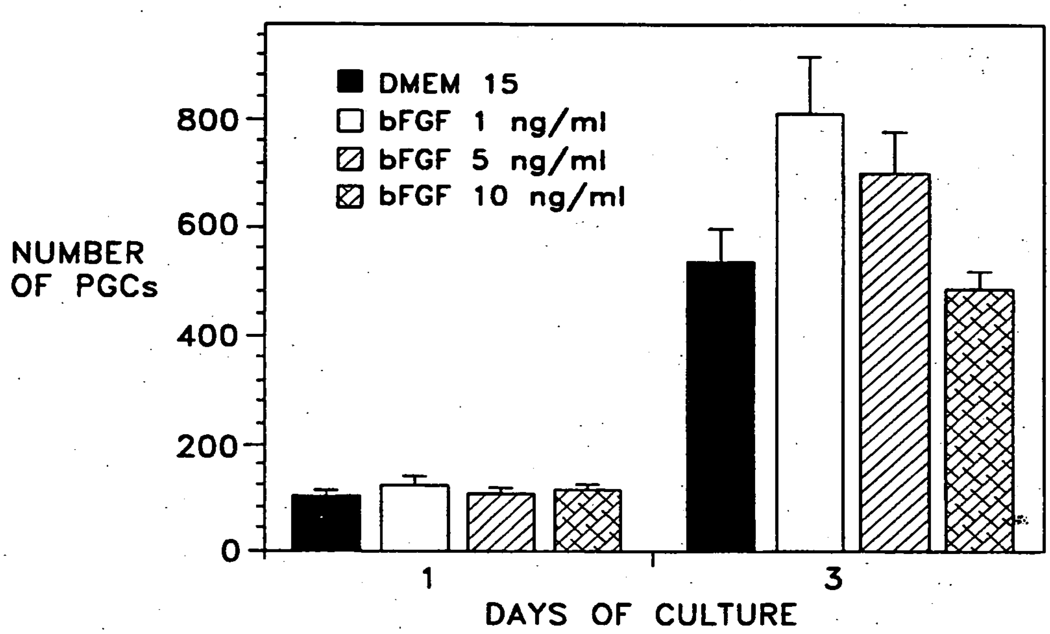
LIF 2000 U/ml
LIF 5000 U/ml
1
3
DAYS OF CULTURE

FIG. 2


800
600
400
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0
NUMBER OF PGCs
DMEM 15
bFGF 1 ng/ml
bFGF 5 ng/ml
bFGF 10 ng/ml
1
3
DAYS OF CULTURE

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 93/09536

A. CLASSIFICATION OF SUBJECT MATTER
IPC 5 C12N5/06 A01K67/027

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 5 C12N A01K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CELL vol. 70 , 4 September 1992 , CAMBRIDGE,MASS.,USA pages 841 - 847 MATSUI ET AL 'DERIVATION OF PLURIPOTENTIAL EMBRYONIC STEM CELLS FROM MURINE PRIMORDIAL GERM CELLS IN CULTURE'	1-14, 16-33
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Date of the actual completion of the international search	Date of mailing of the international search report
31 January 1994	22-02-1994

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Sitch, W

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 93/09536

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	DATABASE WPI Section Ch, Week 9319, Derwent Publications Ltd., London, GB; Class B04, AN 93-159486 DONOVAN ET AL 'LONG TERM CULTURE OF PRIMORDIAL GERM CELLS-IN PRESENCE OF FIBROBLAST GROWTH FACTOR AND LEUKAEMIA INHIBITORY FACTOR,USEFUL IN RESEARCH AND PRODN.OF CHIMERIC AND TRANSGENIC ANIMALS' & US,A,7 958 009 (US DEPT HEALTH AND HUMAN SERVICE) 1 April 1993	1-14, 16-33
P,Y	see abstract ---	15
P,X	NATURE vol. 359 , 8 October 1992 , LONDON,GB pages 550 - 551 RESNICK ET AL 'LONG-TERM PROLIFERATION OF MOUSE PRIMORDIAL GERM CELLS IN CULTURE'	1-14, 16-24
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Y	FILE SERVER STN KARLSRUHE,FILE MEDLINE ABSTRACT NO.79173363 & J CELL PHYSIOL, (1979 MAR) 98 (3) 469-74 PRUSS ET AL:'CHOLERA TOXIN STIMULATES DIVISION OF 3T3 CELLS' see abstract ---	15
Y	FILE SERVER STN KARLSRUHE,FILE MEDLINE ABSTRACT NO.85203765 & EXP LUNG RES, (1985) 8 (1) 53-66 LESLIE ET AL:'STIMULATION OF DNA SYNTHESIS IN CULTURED RAT ALVEOLAR TYPE II CELLS' see abstract -----	15

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